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The Precision of Clinical Estriol and Total Estrogen Estimations in Pregnancy Urine

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Summary: 1. A study of the precision of clinical estriol- and total estrogen determinations in late pregnancy urine was carried out in collaboration with 26 clinical laboratories in the Netherlands and one laboratory in Suriname.

2. Ten urine samples were circulated twice with an interval of 2 weeks.

3. It was shown, that repeated analysis of the same sample in the same clinical laboratory in different assays can yield differences in results up to 40%. This interassay variation can be regarded as the main source of uncertainty of results of clinical estriol and total estrogen determinations.

4. The quantitative differences between results of total estrogen methods and a gas chromatographic method, which measures only estriol, were shown to be caused primarily by the lower recovery of the glucuronide of estriol (both native and added) in the latter method.

5. As expected, methods based on the principle of *Ittrich* ((1960), Acta Endocrinol. *35*, 34–48) proved to be more susceptible to the disturbing influence of glucose than a gas chromatographic method that measured estriol specifically.

6. Various recommendations to improve the precision of clinical estrogen determinations in pregnancy urine resulted from this study.

Die Genauigkeit klinischer Bestimmungen von Östriol und Gesamt-Östrogenen im Harn Schwangerer

Zusammenfassung: 1. In Zusammenarbeit mit 26 klinischen Laboratorien der Niederlande und einem in Surinam wurde eine Untersuchung der Genauigkeit von klinischen Bestimmungen von Östriol und Gesamt-Östrogenen im Harn Hochschwangerer durchgeführt.

2. Zehn Harnproben wurden zweimal mit einem Zwischenraum von zwei Wochen in Umlauf gegeben.

3. Es wird gezeigt, daß die wiederholte Analyse derselben Probe im gleichen klinischen Laboratorium bei verschiedenen Bestimmungen Differenzen der Ergebnisse bis zu 40% ergeben kann. Die Variation von Serie zu Serie kann als Hauptursache für die Unsicherheit der Ergebnisse von klinischen Bestimmungen des Östriols und der Gesamt-Östrogene angesehen werden.

4. Es wird gezeigt, daß die quantitativen Unterschiede zwischen den Ergebnissen von Methoden für Gesamt-Östrogene und einer gaschromatographischen Methode, die nur Östriol mißt, vor allem durch eine niedrige Wiederfindung von ursprünglichem oder zugefügtem Östriol in Glucuronidform bei der Gaschromatographie bedingt sind.

5. Wie zu erwarten war, zeigten sich Methoden nach dem Prinzip von *Ittrich* ((1960), Acta Endocrinol. *35*, 34–48) anfälliger gegen den Störeinfluß von Glucose als eine Östriol spezifisch messende gaschromatographische Methode.

6. Aus dieser Untersuchung ergeben sich verschiedene Empfehlungen zur Verbesserung der Genauigkeit klinischer Bestimmung von Östrogenen im Harn Schwangerer.

Introduction

A study of the precision of estriol- and total estrogen-determinations in pregnancy urine was carried out by the National Institute of Public Health in The Netherlands in close cooperation with 26 clinical laboratories in The Netherlands and one laboratory in Suriname. The object of this study was to obtain information on:

1. intraassay variation;
2. interassay variation in the same laboratory;
3. differences between results obtained with the same method in different laboratories;
4. differences between results obtained by different methods;
5. possible disturbing influences of glucose and/or dilution of the urine.

Originally 48 laboratories participated to the study, but as these 48 participants used 19 different analytical methods, the statistical analysis had to be confined to results obtained with the 3 methods which were used by 5 or more laboratories.

Material and Methods

The composition of the urine samples used is shown in table 1.

The same set of samples was circulated twice, with an interval of 2 weeks. In order to ensure strict objectivity, a different coding of the samples was used per participant and per set; thus a comparison of the results was only possible by the originators of the study.

The samples were prepared in one working day (day 1) using a pool of pregnancy urine collected on the previous day (day 0). The urine pool was not centrifuged and no preservative was added. The estriol-16-glucuronide added to samples 3, 4, 5 and 6 (see table 1) was purchased from Ikafarm (Israël).

The samples were stored at 4°C. On the following day (day 2) set I was dispatched; set II was distributed 13 days later (= day 15). In the intervening period the results of set I had been received by the originators of the study[1]). The pH of the samples stored at 4°C was measured repeatedly during the period of experimentation and was found to be 8.5–8.6.

Tab. 1. Composition of the samples

Number of sample	Composition of sample
1 and 2	pregnancy urine 5* (sample)
3 and 4	pregnancy urine 5 + 20 μmol/l estriol-16-glucuronide
5 and 6	pregnancy urine 5 + 40 μmol/l estriol-16-glucuronide
7 and 8	pregnancy urine 5 + 80 g/l glucose
9 and 10	½ volume pregnancy urine 5 and ½ volume male urine 2*

* In a former study (not to be discussed further here), pregnancy urines 1, 2, 3 and 4, and male urine 1 were analysed.

[1]) In one case ("Van Kessel 7") however the delay between dispatch and receipt of results was as long as 22 months. The results are included in table 2, but *not* in the statistical calculations.

The methods used by 5 or more participants per method were the following: (n = number of participating laboratories).

1. *Brombacher, Gijzen & Verheesen* (1968) (1). n = 11
2. *Van de Calseyde, Scholtis, Schmidt & Kuypers* (1969) (2). n = 9
3. *Van Kessel, Seitzinger, Schreurs & Versteeg* (1969) (3). n = 7

The method of *Brombacher et al* is based on the work of *Ittrich* (4). Pregnancy urine is diluted with water (1 : 99). Diluted urine (0.5 ml) is pipetted into a tube, followed by 0.5 ml water and 2 ml 960 g/kg sulphuric acid containing 10 g/l hydroquinone. The reaction mixture is heated in a boiling water bath for 40 minutes. After cooling in ice, 3.75 ml water are added, the contents of the tube are mixed, then cooled and extracted with 4 ml of an ice-cold 20 g/l solution of *p*-nitrophenol in chloroform. After centrifugation, the upper layer is removed and the fluorescence is measured with a Vitatron fluorimeter: excitation 359 nm, fluorescence 577 nm.

The main difference between the methods '*Brombacher*' and '*Van Kessel*' is, that the latter method is fully mechanized. The relatively simple apparatus used is described in the publication of *Van Kessel* et al (1969). The sulphuric acid reagent is made by dissolving hydroquinone (20 g) in water (280 ml) followed by the addition of 960 g/kg sulphuric acid, with cooling and stirring, to a volume of 1 l. *p*-Nitrophenol in dichloroethane (20 g/l) is used for extraction. The fluorimeter (Vitatron) is equipped with a mercury lamp, and the spectral line of 546 nm, for excitation of fluorescence is selected by means of an interference filter; the secondary light is measured after passing a filter of 564 nm.

The method of *Van de Calseyde et al* makes use of enzymatic hydrolysis of the estriol conjugates with the juice of *Helix pomatia* (0.1 ml/ml of urine at pH 4.6; incubation for 30 minutes at 62°C). After extraction with chloroform and evaporation of the solvent, 50 μl of bis-trimethylsilylacetamide and 20 μg of trimethylchlorosilane are added. After 5 minutes 1 μl of reaction mixture may be used for gas liquid chromatography (Micro-Tec G. C. 2000 MF, with a dual ionization detector). The stainless steel U column (diameter 4 mm) is packed with 3% O.V.-1 (w/w) on gaschrom Q 100–200 mesh (Applied Science Lab.). The column temperature program is as follows: initial temperature 240°C for 3 minutes; heating (7.5°/minute) for 3 minutes; heating (2°/minute) for 3 minutes; the final temperature is 270°C. The temperature of injector and detector is 285°C.

Statistical analysis of results

Unless stated otherwise, the statistical evaluations were based on straightforward applications of the general principle of analysis of variance. Two other statistical tests, viz. *Tukey*'s test for multiple comparisons between group means and *Dixon*'s outlier test, have been taken from l.c. (5).

Results

The results submitted by the participants are shown in table 2. They are expressed in μmol of estriol per liter.

The numbering of the participants is the same as in table 3, which deals with the coefficients of variation.

Unfortunately not all participants managed to adhere to our request to measure the series of 10 samples within the same assay. Under this circumstance a division of the results into a

Tab. 2. Results obtained by 26 different clinical laboratories using 3 different methods.

Method	No. of participant	No. of set	Results in µmol/l (samples 1–10) 1	2	3	4	5	6	7	8	9	10
Brombacher et al (1)	1*	I	73	73	97	101	114	118	59	55	28	31
		II	73	75	101	101	121	123	47	55	33	38
	2	I	83	85	95	98	125	125	50	50	38	38
		II	84	76	92	101	112	119	40	50	38	36
	3*	I	70	83	96	84	100	108	56	57	39	36
		II	73	70	86	80	92	103	42	43	34	33
	4	I	99	107	136	158	167	176	105	107	57	52
		II	166	144	170	173	207	235	149	132	73	69
		II (revised)	83	82	105	103	126	117	77	80	42	43
	5*	I	93	100	125	113	111	132	90	100	45	48
		II	100	97	100	112	185	131	115	90	37	35
	6	I	114	100	107	121	153	144	75	67	53	53
		II	121	114	128	137	210	120	90	94	64	90
		II (revised)	78	81	97	94	117	107	61	58	38	33
	7	I	96	85	114	113	125	125	117	86	37	56
		II	90	92	99	105	120	124	104	94	67	47
	8	I	77	73	83	90	79	71	47	38	38	35
		II	42	46	76	52	67	61	27	33	17	30
	9*	I	86	37	56	113	125	114	85	125	117	96
		II	114	62	139	182	111	116	96	105	39	34
	10	I	.	133	168	244	92	149	88	183	71	46
		II	60	76	131	195	88	42	86	56	40	86
	11	I	190	115	168	124	235	165	57	56	36	56
		II	200	480	331	980	401	0	90	110	93	112
Van de Calseyde et al (2)	1*	I	61	56	83	74	82	80	53	56	34	30
		II	68	74	91	85	99	102	63	57	33	39
	2*	I	71	56	84	88	112	106	67	62	28	34
		II	70	69	79	90	102	102	62	61	33	40
	3	I	64	55	54	71	69	101	33	35	49	33
		II	48	42	56	60	70	79	45	37	25	28
	4*	I	64	42	74	45	85	80	48	58	24	31
		II	58	60	78	74	87	93	57	56	26	28
	5*	I	36	63	64	66	81	93	42	59	13	29
		II	55	68	82	85	71	96	64	59	35	38
	6	I	53	46	.	55	22	74	48	38	25	22
		II	51	52	63	67	80	75	47	45	25	27
	7	I	30	35	42	22	55	43	37	28	59	20
		II	51	66	81	76	88	82	59	60	34	27
	8*	I	52	51	35	50	47	31	16	42	24	9
		II	40	38	22	31	70	37	23	34	22	25
	9*	I	13	9	7	7	6	8	11	16	4	3½
		II	6	4	6	7	6	6	10	9	4	3½
Van Kessel et al (3)	1	I	94	98	113	116	132	131	.	.	49	48
		II	110	106	129	127	153	153	.	.	49	51
	2	I	78	83	89	91	111	113	65	61	43	43
		II	85	85	92½	100	114½	114½	63	63	40½	40½
	3	I	83	86	104	108	129	130	70	70	40	39
		II	88	88	105	108	129	128	78	90	36	37
	4	I	82	84	99	101	119	122	88	92	41	41
		II	93	86	99	99	118	114	86	85	39	46
	5	I	88	83	95	100	128	109	71	70	43	43
		II	85	90	104	101	122	123	70	71	45	39
	6	I	140	115	175	140	160	166	36	42	66	66
		II	105	102	135	125	155	150	25	.	50	50
*Van Kessel*** et al (3)	7	I	72½	72½	91	84½	98	109	70	71	29	26½
		II	63	72½	101	92	110	107	82	76	32	35

*) = group b (group a: unmarked)
.) = no results received
**) = these results were obtained with samples which were kept deep frozen by the participant for *22 months* before analysis. They are *not* included in the statistical calculations

N.B. Participants "Brombacher 4 and 6" submitted, on a later date, revised results for series II, obtained after a slight modification of their analytical procedure. These results are included in the table as II (revised).

Tab. 3. Estimated coefficients of variation [%] within sample duplicates of sets I and II.

Method	No. of participant	Estimated coefficient of variation [%]	
		group a	group b
Van Kessel et al (3)	1	2.0	.
	2	2.7	.
	3	3.9	.
	4	4.5	.
	5	5.4	.
Brombacher et al (1)	1	.	5.6
	2	6.2	.
	3	.	6.4
Van de Calseyde et al (2)	1	.	6.7
Brombacher et al (1)	4	6.9	.
Van Kessel et al (3)	6	8.1	.
Van de Calseyde et al (2)	2	.	8.9
Brombacher et al (1)	5	.	11.5
	6	14.1	.
	7	14.7	.
Van de Calseyde et al (2)	3	15.9	.
	4	.	16.8
Brombacher et al (1)	8	17.9	.
Van de Calseyde et al (2)	5	.	26.0
	6	31.4	.
Brombacher et al (1)	9	.	32.6
Van de Calseyde et al (2)	7	32.7	.
	8	.	41.8
Brombacher et al (1)	10	> 50	.
	11	> 50	.
Van de Calseyde et al (2)	9	rejected	rejected

The standard error is obtained by dividing the coefficient of variation by 5. Only the results of group a were obtained according to the rule that measurement of series of 10 samples should take place within one assay.

N.B. Participants "Brombacher 4 and 6" submitted, on a later date, revised results for series II, obtained after a slight modification of the analytical procedure: the estimated coefficients for these revised results are: "Brombacher 4": 1,5%, "Brombacher 6": 2,9%.

group a and a group b was necessary. Only the former results (group a) were obtained in accordance with our request that samples belonging to the same set be measured in the same assay.

Intraassay variation

As the standard deviation for the 10 sample replicates per participant (table 2) was found to be roughly proportionate to the level of the results measured, it seemed plausible to work with coefficients of variation.

The coefficients of variation were calculated as follows: For the decimal logarithms of the results listed in table 2, the proper standard deviations were calculated for the variation within sample replicates.

The antilogarithm of each standard deviation minus 1 is, to a first order approximation, equal to the estimate of the coefficient of variation for the participant under consideration.

Table 3 gives the calculated coefficient of variation per participant.

Participant "Van de Calseyde No. 9" submitted extremely low results (table 2); these results had to be rejected by the statistician.

The intraassay variation was generally low in the group working with the mechanized *Van Kessel* method and in general more marked in the results obtained with the two non-automated methods.

Interassay variation

This effect was studied on the basis of the total percentual recovery. This figure can be calculated from the results obtained, if it can be shown that an adequate proportionality exists between the percentual recovery of added estriol (= b) on the one hand and the mean result of the samples 1 to 6 (pregnancy urine 5 + 20 μmol estriol), which can be considered as a measure for native estrogen, on the other hand ($\bar{y}$).

For $\bar{y}$ (= the mean of samples 1 to 6) we have 2 values for each participant, i.e. from the first and the second set of results. For b (= percentual recovery of added estriol) two values can be obtained for each participant per set namely:

1. results of samples 3 + 4 minus results of samples 5 + 6
2. results of samples 1 + 2 minus results of samples 3 + 4 (see table 1).

The 2 values for b per set per participant were tested for equality, after which the mean b per set per participant was calculated.

Thereupon the quotient y/b (=Q) per set was calculated for each participant; after testing for equality (set I and set II) the mean value for Q per participant was calculated. If the assumption that an adequate proportionality exists between the recovery of native estrogen (of which y is a measure) and the percentual recovery of added estriol (of which b is a measure) is correct, then all Q's should be statistically equal, at least for results obtained by the same method or by methodologically related (equivalent) methods such as '*Van Kessel*' and '*Brombacher*'.

The '*Van de Calseyde*' method however measures the estriol content, whereas the two other methods measure the total estrogen content. Lower values for y could therefore be expected with the '*Van de Calseyde*' method, as in fact were found (fig. 1).

The b value, however, (= the percentual recovery of added estriol) should be statistically equal for the 3 methods. This is, however, not the case; the b values are generally lower in the '*Van de Calseyde*' method. Consequently it could not be demonstrated statistically, that the difference between the specific estriol method on the one hand, and the two other total estrogen methods on the other hand can be ascribed entirely or largely to the fact that the *Van de Calseyde* method measures estriol, while the other 2 methods measure the total estrogen content: there is in fact no statistically significant difference between the Q values obtained with the 3 methods.

In the further statistical analysis of the results, no distinction was made, therefore, between the results of the 3 methods. The mean Q was used to construct the line in figure 1. With the aid of this line, the ideal y (= the native estrogen content of pregnancy urine 5 + 20 μmol estriol) could be determined.

In figure 1, results submitted by participant "Van de Calseyde No. 9" and "Brombacher Nos. 10 and 11" have been eliminated in the view of very poor intraassay reproducibility (see tab. 3); the results of "Van de Calseyde 6" have been eliminated on account of unacceptable incompleteness (see tab. 2).

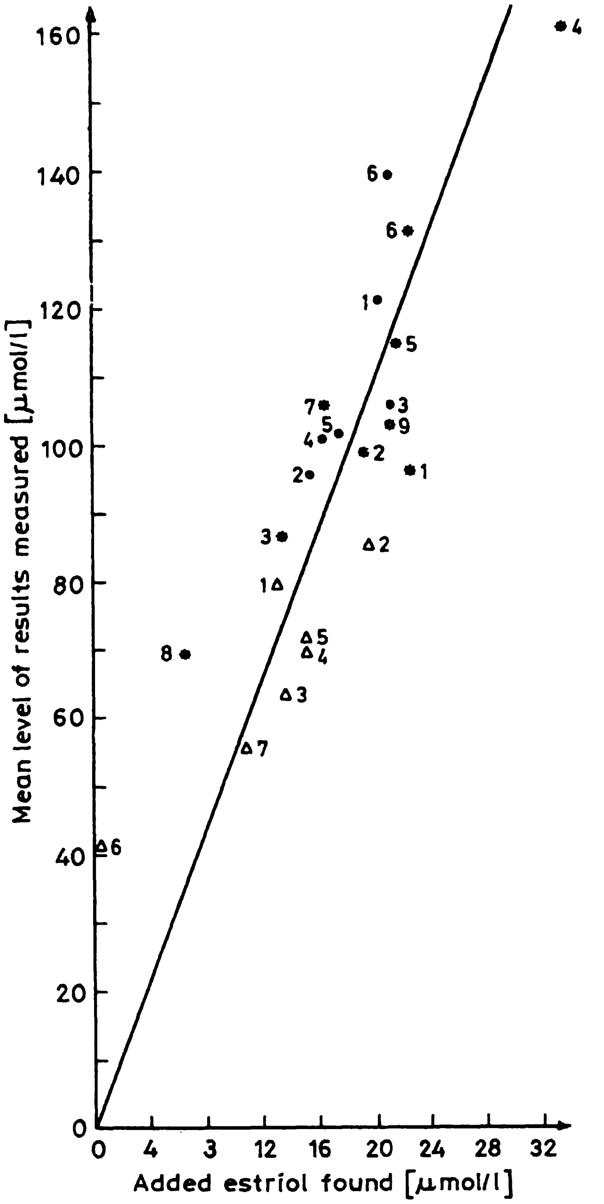

Fig. 1. Proportionality between recovery of estriol added (b) and mean level of results measured (y).

Abscissa: b = mean results [(urine 3 + urine 4) – (urine 1 + urine 2)] and [(urine 5 + urine 6) – (urine 3 + urine 4)]

Ordinate: y = mean result for samples 1–6

• = Method of *Van Kessel* et al. (3)

* = Method of *Brombacher* et al. (1)

△ = Method of *Van de Calseyde* et al. (2)

The figure obtained for the ideal y, which we need in order to calculate the percentual total recovery per set as submitted by each participant, is equal to 110 μmol (estriol equivalents). The individual y's per set per participant are expressed as a percentage of the ideal y; thus for each participant 2 values for the percentual total recovery were obtained. In figure 2 the difference in the percentual total recovery y (set 1 – set 2) per participant is plotted on the abscissa; this difference is either positive or negative.

The number of participants has been plotted on the ordinate, again with a division into group a and group b (compare table 3). The fact that the range of the interassay variation in group a exceeds that in group b is entirely in accordance with expectations. The fairly normal distribution shows that, statistically, there is no detectable difference in estrogen content between the samples of set I and set II. The fluctuation in percentual recovery between two assays in the same laboratory can be important (up to 40 percent).

The results submitted by participants "Van de Calseyde 6 and 9," and of "Brombacher 10 and 11" have been excluded from figure 2 as they were from figure 1.

Interlaboratory variation

Table 4 gives an impression of the differences in percentual total recovery obtained with the same method in different laboratories.

For strictly statistical reasons results with a relatively poor intraassay reproducibility ("Van de Calseyde Nos. 5, 6, 7 and 8"; "Brombacher Nos. 9, 10 and 11", compare tab. 3) had to be left out of consideration in the subsequent statistical procedure. "Brombacher No. 8", with a comparatively low percentual recovery, differs significantly from the other results in the "Brombacher group" (*Dixon*'s test (5)). Subsequently information was received about the special position of the laboratory concerned, which justifies the exclusion of this result from the statistical calculation.

The interlaboratory variation of the 3 groups under investigation appears to exceed significantly the expectations based on the two sources of variation (intra- and interassay variation), that have already been discussed. This leads to the conclusion, that a significant interlaboratory variation is present.

The statistical calculations carried out were as follows: For each participant belonging to group a, the square of the difference between the two estimated percentual total recoveries for set I and set II is calculated; the sum of these squares divided by 24 (= 2x number of participants considered) equals 130, which is a measure of the variance due to both intraassay and interassay variation.

From the total percentual recoveries per participant (tab. 4) we calculated, for each of the 3 methods separately, the sum of squared deviations from the mean. Pooling the three sums of squares, the figure 315 is obtained, a value to which 14 degrees

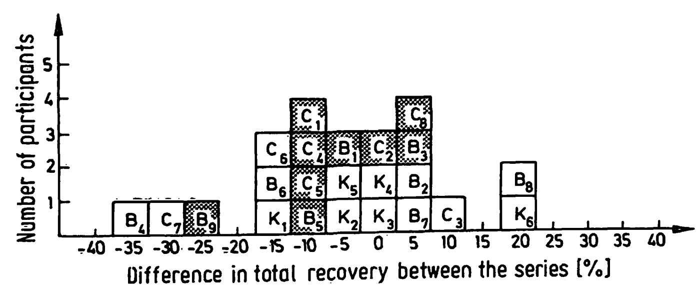

Fig. 2. Interassay variation.

K = Method of *Van Kessel* et al. (3)

B = Method of *Brombacher* et al. (1)

C = Method of *Van de Calseyde* et al. (2)

□ = group a

▨ = group b

Only for group a could it be assumed with reasonable certainty that determinations were really made within two assays.

Tab. 4. Estimated percentual total recoveries per set.

Method	No. of participant	Total recovery [%] Set I	Set II	Mean total recovery per participant [%]
Brombacher et al (1)	1*)	88	91	$90^1/_2$
	2	93	89	91
	3*)	83	77	80
	4	129 (.)	167 (94)	148
	5*)	103	111	106
	6	113 (.)	127 (88)	120
	7	101	96	$98^1/_2$
	8	72	54	63
	9*)	81	111	91
Van de Calseyde et al (2)	1*)	67	79	73
	2*)	79	78	$78^1/_2$
	3	63	54	$58^1/_2$
	4*)	60	69	$64^1/_2$
	5*)	62	70	68
	6	46	59	$57^1/_2$
	7	35	68	$51^1/_2$
	8*)	41	36	$38^1/_2$
Van Kessel et al (3)	1	105	109	107
	2	86	91	$88^1/_2$
	3	98	99	$98^1/_2$
	4	93	93	93
	5	92	96	94
	6	137	118	$127^1/_2$

*) group b. Only the results of group a (unmarked) are obtained according to the rule that measurement per set of 10 samples should take place within one same assay.
( ) revised results. (.) no result.

of freedom are to be attached and which is significantly greater than $^1/_2$ x the value 130 just mentioned (F-ratio; $P < 0.05$). Hence the estimated interlaboratory variance is significantly positive and equals $315 - {}^1/_2 \times 130$, viz. 250. This implies an (extra) standard deviation of roughly 16 units for each one of the total mean recoveries considered.

Differences between results obtained by different methods

Statistical calculation, using *Tukey*'s test (5) at a confidence level of 5%, revealed no statistical difference between the percentual total recoveries of the "Brombacher" group and those of the "Van Kessel group", provided that the results of "Brombacher No. 8" are excluded. The special position of this participant, justifying the exclusion, was mentioned above. A significant difference however was present between the results obtained by the method based on the *Ittrich* principle (*Brombacher* and *Van Kessel*) on the one hand and the results obtained by the gas chromatographic estriol method (*Van de Calseyde*) on the other hand. As already mentioned above, this difference cannot be ascribed entirely or largely to the fact that the *Van de Calseyde* method measures estriol, whereas the other methods measure the total estrogen content. In the *Van de Calseyde* method, not only the y-value (a measure for the recovery of native estrogen) is lower; the same is true of the b-value which is a measure for the recovery of added estriol. So a lower recovery of native and added estriol in the *Van de Calseyde* method must be considered as the principal origin of the observed difference.

The role of glucose and of dilution of the urine

The conclusions on the result obtained with the glucose-containing sample and the diluted pairs are summarized in table 5.

For the glucose-containing sample pair, we tested the extent to which the mean result differed significantly from the observed estriol content of the original urine per dispatched series and per participant. An analogous procedure was applied to the diluted sample pair (original urine diluted 1 : 2), accounting for a factor 2. In the methods based on the *Ittrich* principle, glucose proved to be a disturbing factor. We may conclude that dilution of the urine did not interfere with the analytical methods.

Discussion

Our study was carried out in order to pave the way for adequate quality control; it resulted in information as to the causes of unreliable results as well as in suggestions for improvement.

1. The most important result is that repeated blind analysis of the same sample in the same laboratory

Tab. 5. Results obtained for the special sample pairs 7,8 and 9,10.

Method	Samples 7,8 (glucose) number of participants	number of participants with significant results S−	S+	Samples 9,10 (diluted) number of participants	number of participants with significant results S−	S+
Brombacher et al (1)	9	4	0	9	0	0
Van Kessel et al (3)	5	2	0	6	1	0
Van de Calseyde et al (2)	8	0	0	8	0	1

S− = significantly lower than original urine (7,8) or $^1/_2 \times$ original urine (9,10)
S+ = significantly higher than original urine (7,8) or $^1/_2 \times$ original urine (9,10)
Participants "Van de Calseyde 9" and "Brombacher 10 and 11" have been excluded from the calculation in view of very poor reproducibility (see table 3).

can yield differences in results in different assays, which substantially and significantly exceed those which could be explained by the reproducibility within one assay. This source of uncertainty may effect in an unacceptable way the diagnostic value of serial estriol (or total estrogen) determinations in one patient. According to *Kuss* (6) the practicability of estrogen determinations in pregnancy urine is impaired, if the day to day coefficient of variation exceeds 5 per cent.

Strict control of experimental conditions in the individual laboratories is therefore recommended in order to diminish the interassay variation.

2. With respect to differences between results obtained with the same method in different laboratories the following can be said:

   Even during the investigation itself a need for a more detailed description of methods was felt by several participants. The desirability of the use of a common standard preparation (preferably estriol-16-glucuronide) was also emphasised. There was also the question of the quality of reagents, which was by no means the same in all the participating laboratories. It was felt that several measures could be taken in order to diminish the interlaboratory differences between laboratories working with the same or comparable methods.

3. The difference in results obtained by methods based on the *Ittrich* (4) principle on the one hand and the specific gas chromatographic method for estriol on the other hand, which was not exclusively or largely attributable to the difference between estriol- and total estrogen content, may be the result of incomplete enzymatic hydrolysis (see *Graef & Fuchs* (7)) and/or losses during the purification procedure. If it is a question of variable losses, the diagnostic value of serial estriol determination in one patient may be impaired. Recovery experiments with conjugated and free estriol could be used to determine the origin of these losses.

4. It is certain, that the present study is quite incomplete as far as disturbing factors are concerned. It is known that bile pigments (*Adlercreutz & Schauman*, (8)) and pharmaceuticals (*Sele & Frandsen*, (9)) may interfere in the *Kober* reaction (10).

   Nevertheless, it is possible to draw the conclusion, that the *Van Kessel* and *Brombacher* modification of *Ittrichs* method are susceptible to the disturbing influence of glucose, whereas the gaschromatographic *Van de Calseyde* method is not. Apparently, none of the participants working with methods based on *Ittrich*'s work made use of methods to destroy or to eliminate glucose (*Worth* (11), see also *Simkins & Worth* (12); *Kuss* (13)).

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References

1. Brombacher, P. J., Gijzen, A. J. H. & Verheezen, P. E. (1968), Clin. Chim. Acta *20*, 360–361.
2. Van de Calseyde, J. F., Scholtis, R. J. H., Schmidt, N. A. & Kuypers, A. M. J. (1969), Clin. Chim. Acta *25*, 345–349.
3. Kessel, H. van, Seitzinger, R., Schreurs, J. & Versteeg, M. (1969), Ned. T. Verlosk. *69*, 81–90.
4. Ittrich, G. (1960), Acta Endocrinol. *35*, 34–48.
5. Miller, Jr., G. M. (1966), Simultaneous Statistical Inference. Mc. Graw-Hill Book Company, New York.
6. Kuss, E. (1970), Z. Klin. Chem. Klin. Biochem. *8*, 339–344.
7. Graef, V. & Fuchs, M. (1975), Z. Klin. Chim. Klin. Biochem. *13*, 163–167.
8. Adlercreutz, N. & Schauman, K. O. (1964), Acta Endocrinol. *46*, 230–234.
9. Sele, V. & Frandsen, V. A. (1967), Acta Obstetr. Gynecol. Scand. *46*, suppl. *9*, 43–47.
10. Kober, S. (1931), Biochem. Z. *239*, 209–212.
11. Worth, H. G. J. (1973), Clin. Chim. Acta *49*, 53–59.
12. Simkins, A. & Worth, H. G. J. (1975), Ann. Clin. Biochem. *12*, 233–243.
13. Kuss, E., Methods of Hormone Analysis, p. 463–468. Georg Thieme Verlag Stuttgart/John Wiley and Sons, New York, 1975.

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